

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



(19)

Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) EP 0 748 439 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
21.07.1999 Bulletin 1999/29

(51) Int Cl. 6: **G01N 1/28, G02B 21/32,
G01N 33/483**

(21) Application number: **95911965.2**

(86) International application number:
PCT/US95/02432

(22) Date of filing: **01.03.1995**

(87) International publication number:
WO 95/23960 (08.09.1995 Gazette 1995/38)

(54) ISOLATION OF CELLULAR MATERIAL UNDER MICROSCOPIC VISUALIZATION

ISOLIERUNG VON ZELLMATERIAL MITTELS MIKROSKOPISCHER DARSTELLUNG

ISOLEMENT DE MATERIEL CELLULAIRE SOUS MICROSCOPE

(84) Designated Contracting States:
**AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL
PT SE**

Designated Extension States:
LT SI

(30) Priority: **01.03.1994 US 203780**

(43) Date of publication of application:
18.12.1996 Bulletin 1996/51

(73) Proprietor: **GOVERNMENT OF THE UNITED
STATES, represented by THE SECRETARY OF
THE DEPARTMENT OF HEALTH AND HUMAN
SERVICES
Washington, DC 20201 (US)**

(72) Inventors:

- **LIOTTA, Lance, A.
Potomac, MD 20854 (US)**

- **ZHUANG, Zhenping
Rockville, MD 20852 (US)**
- **BUCK, Michael, R.
Bethesda, MD 20814 (US)**
- **STETLER-STEVENSON, William, G.
Kensington, Maryland 20895 (US)**
- **LUBENSKY, Irina, A.
Silver Spring, MD 20906 (US)**
- **ROTH, Mark, J.
Silver Spring, MD 20904 (US)**

(74) Representative: **Portal, Gérard et al
Cabinet Beau de Loménie
158, rue de l'Université
75340 Paris Cédex 07 (FR)**

(56) References cited:

EP-A- 0 539 888	DE-B- 1 074 885
US-A- 4 144 760	US-A- 4 619 899
US-A- 4 749 868	US-A- 5 218 645
US-A- 5 252 487	

EP 0 748 439 B1

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Description**Technical Field**

5 [0001] The present invention relates to methods and devices for the analysis of cellular samples. More particularly, the present invention relates to methods and devices for the microdissection and analysis of cellular samples which may be used in combination with a number of different technologies that allow for analysis of enzymes, mRNA and DNA from pure populations or subpopulations of particular cell types.

Background Art

10 [0002] Many diseases are now understood at the molecular and genetic level. Analysis of such molecules is important for disease diagnosis and prognosis. Previous methods for direct extraction of cellular tissue material from a tissue sample are limited because the extraction reflects only the average content of disease associated markers. In reality, 15 tissues are very heterogeneous, and the most diagnostic portions of the tissue may be confined to a few hundred cells or less in a lesion.

15 [0003] The molecular study of human tumors is currently limited by the techniques and model systems available for their characterization. Studies to quantitatively or qualitatively assess proteins or nucleic acid expression in human tumor cells are compromised by the diverse cell populations present in bulk tumor specimens. Histologic fields of 20 invasive tumor typically show a number of cell types including tumor cells, stromal cells, endothelial cells, normal epithelial cells and inflammatory cells. Since the tumor cells are often a relatively small percentage of the total cell population it is difficult to interpret the significance of net protein or nucleic acid alterations in these specimens.

25 [0004] The processes of tumor invasion and metastasis depend upon increased proteolytic activity of invading tumor cells. Matrix metalloproteinases, cathepsins B, D, and L, and plasminogen activator have been implicated in the metastatic cascade. Cathepsin D has been suggested to be an independent marker of prognosis in breast cancer. Several 30 lines of correlation evidence support the concept that proteases are important in tumor invasion including: increased protease activity and/or altered subcellular distribution of proteases in highly metastatic tumor cell lines, increased protease expression in invasive human tumors as determined by both immunohistochemistry and assays of tumor tissue homogenates, and increased mRNA levels in human tumors. All of these techniques have generated important information regarding protease expression in human tumors, however, they have not provided definitive evidence that proteases are up-regulated in specific regions where tumor invasion is occurring.

35 [0005] Studies of human tumor cells in culture do not account for the complex interactions of the tumor cells with host cells and extracellular matrix, and how they may regulate tumor cell protease productivity or activation. Immunohistochemical staining allows one to examine enzyme distribution in regions of tumor invasion, however, results vary with tissue fixation and antibody-antigen affinity, and provide only a semi-quantitative assessment of protein levels. Furthermore, quantitative interpretation of staining results is complicated by the variability of staining patterns within 40 tissue sections, subjective evaluation of staining intensity, and the difficulty in interpreting the significance of stromal staining. In addition, many antibodies utilized in the study of proteases do not differentiate pro-enzyme from active enzyme species. Assays of enzyme or mRNA levels from homogenates of human tumors does not account for either the mixed population of cells within the specimens, or the concomitant pathophysiologic processes which may occur in the tissue.

45 [0006] The present invention is directed to a sampling technique for the microdissection of cellular tissue which allows for examination of specific histologic fields and cell populations within those fields.

[0007] EP-A-0 539 888 relates to a device for selecting cells and the like: first of all, cells and the like are arranged on a support member in a single layer. A detector then detects each optical property of the cells and the like, and detectors detect each position of the cells and the like with respect to the structure. Data obtained by the detectors are stored in a memory device. According to the stored data, a control device moves a pickup member relatively in the three dimensional directions with respect to the support member. The pickup member picks up any of the cells and the like which has a specific optical property via an adhesive. Each of the specific cells and the like which adheres to the pickup member is releasable from the pickup member. This document does not teach direct extraction of cellular material from a tissue sample. However, such a sample presents problems which are not encountered in the reference method. Furthermore, this document does not suggest a step of analyzing the extracted cellular material at a molecular level.

[0008] The German patent, DE-B-1 074 885, relates to a procedure for selecting microfossils from sediments or finely crushed rock.

[0009] US patent US-A-4,749,868 relates to a method of processing a sample containing water under scanning electron microscope and an apparatus: fragments are extracted from a raw sample containing water such as a fungi attached to a plant. The sample is rapidly frozen and cut under vacuum largely and at random. Water contained in the

sample is sublimated and the sample is coated with conductive material. While observing the sample by a scanning electron microscope, useful fragments are extracted from the sample by a micromanipulator. This document however is directed to the use of a "needle", and furthermore, this document neither teaches nor suggests any analysis at molecular level.

5

Disclosure of the Invention

[0010] It is according one object of the present invention to provide a method of identifying specific cells in cellular tissue sample.

10 [0011] Another object of the present invention is to provide a method of direct extraction of specific cells from a cellular tissue sample.

[0012] It is a further object of the present invention to provide an automated method of identifying specific cells in cellular tissue sample.

15 [0013] A further object of the present invention is to provide an automated method of direct extraction of specific cells from a cellular tissue sample.

[0014] A still further object of the present invention is to provide a method of obtaining pure cell populations from a cellular tissue samples.

20 [0015] According to these and further objects of the present invention which will become apparent as the description thereof proceeds, the present invention provides for a method of direct extraction of cellular material from a tissue sample which involves :

- a) providing a slide-mounted tissue sample having a plurality of zones of cells;
- b) forming an image field of cells of said tissue sample utilizing a microscope;
- c) identifying at least one zone of cells of interest in said image field of cells, the at least one zone of cells of interest having a different type of cell than an adjacent zone of cells;
- d) extracting by microdissection said at least one zone of cells of interest from said tissue sample by :

25 d1) i) contacting the at least one zone of cells of interest with an adhesive-tipped probe which has a contact surface area which is equal to or no greater than a surface area of the at least one zone of cells of interest, wherein the relative adhesive force of the adhesive tipping said probe is greater than the adhesive force of the one zone of cells of interest on said slide; and then
30 d1) ii) retracting the adhesive-tipped probe from the position of contact with the tissue sample, thereby causing said at least one zone of cells of interest in the image field of cells attached to the contact surface area of the adhesive-tipped probe to be pulled away by microdissection.

35

before finally:

analyzing said extracted at least one zone of cells of interest.

40

Brief Description of Drawings

[0016] The present invention will be described with reference to the attached drawings which are given by way of non-limiting examples only, in which:
45 [0017] Figure 1 is a functional system diagram which shows how a tissue sample is microscopically imaged, displayed on a display monitor, and how a region of the imaged sample is selected and identified for subsequent microdissection and analysis.

[0018] Figures 2a-2c are a series of functional system diagrams which show how a zone of tissue sample is extracted from the slide-mounted tissue sample according to one embodiment of the present invention.
45 [0019] Figure 3 is a schematic illustration of an alternative device for extracting sample zones from the slide-mounted tissue sample.

50 [0020] Figures 4a and 4b are schematic diagrams of a manual extraction tool manipulator which can be used together with the extraction device of Fig. 3 according to the present invention.
[0021] Figure 5 is a functional system diagram which shows how a zone of sample tissue can be directed to an appropriate analysis protocol.

55 [0022] Figure 6a and 6b show the expression of MMP-2 in ten invasive colon carcinoma cases (Fig. 6a) and in five cases of invasive breast carcinoma (Fig. 6b) as compared to normal colonic mucosa from the same patients.
[0023] Figure 7 shows SSCP analysis of MMP-2 activation site.

Best Mode for Carrying out the Invention

[0024] The present invention is directed to a method of analyzing cellular material on a molecular or genetic level which involves: visualizing a field of cells in a tissue sample under a microscope, contacting an identified area with a surface which simultaneously dissolves, extracts and/or retains a cellular material of interest, and transferring the cellular material of interest to a suitable analysis system. The present invention is particularly applicable to the analysis of local tissue enzymes, antigens, DNA, RNA, and the like.

[0025] The present invention is also directed to a fully automated system whereby a tissue can be visualized on a screen, so that a precise field of cells of interest can be identified by a variety of labels, circumscribed, and then be automatically extracted and analyzed.

[0026] Figure 1 is a functional system diagram which shows how a tissue sample is microscopically imaged, displayed on a display monitor, and how a region of the imaged sample is selected and identified for subsequent microdissection and analysis. As depicted in Fig. 1, a tissue sample 1 is provided on a glass slide 2 for microscopic examination and imaging. The sample tissue 1 can be fixed on the glass slide 2 according to any conventional method, including attachment to the glass slide 2 with an agarose gel, fixing the tissue sample in paraffin, etc.

[0027] The glass slide 2 having the sample tissue 1 mounted thereon is placed on the stage of a microscope. The microscope, generally indicated by reference numeral 3 receives an image of the tissue sample 1. A video camera (not shown) is connected to the microscope 3. The video camera receives the image of the sample tissue 1 from the microscope 3 and displays the image of the tissue sample on a display monitor 4.

[0028] The image of the sample tissue 1 is limited to the "field" of the microscope 3 for any given image. As indicated in Fig. 1, the field of the sample tissue image may include several zones, "A", "B", "C", and "D" of different types of cells which can be optically distinguished by utilizing a suitable dye(s) to stain the tissue sample. For exemplary purposes, Figs. 1 and 2a-2c assume that zone "B" is the zone of cellular material of interest. The image on the display monitor 4 is used by the operator to select and identify one or more zones of the tissue sample 1 which are of interest.

[0029] According to one embodiment of the present invention, after the zone(s) of interest are selected and identified, the operator manually manipulates a device to extract the identified zone(s) from the glass slide 2. The extracted zone(s) of sample material may either include an analysis sample. Otherwise, the identified and extracted zone(s) can include zones which are to be discarded and the remaining zone(s) which are retained on the glass slide 2, can be later analyzed.

[0030] In addition to manual operation which is discussed in more detail below, it is possible, according to another embodiment of the present invention, to utilize the image on the display monitor 4 to select and identify a sample zone (s) whose relative position is determined utilizing a computer which receives a digitized signal of the image from the video camera (or microscope), and which receives a reference position of the stage of the microscope 3 upon which the sample is held. Such positioning detection and recognition systems are conventional in the art and can be readily applied to automate the sample preparation method of the present invention. In this automated embodiment of the invention, the computer which performs the positioning detection and recognizing can also be used to control the movement of the devices discussed below that are used to extract tissue zones, thus automating the sample removal. In addition, the image of the sample can be electronically scanned to automatically identify zones having a predetermined or relevant degree of staining, using known techniques and devices. Thus, in a preferred embodiment, a computer could be used to select and identify zones of interest and the relative position of such zones, for manipulating a device to remove such zones in an automated manner.

[0031] The embodiment of the invention depicted in Figs. 2a-2c utilize a contact probe 5 which has an adhesive/extraction reagent 6 on the tip thereof. A suitable adhesive/extraction reagent can include a mixture of piccolyte and xylene. In Fig. 2a the contact probe 5 is positioned either manually or by computer control so as to be above and aligned with the sample zone ("B") to be extracted. As can be readily understood from Fig. 2a, the surface area of the contact probe tip (and adhesive/extraction reagent) needs to be about equal to, and no greater than, the surface area of the zone to be extracted. Otherwise, excessive removal of adjacent tissue zones will occur.

[0032] Once the tip of the contact probe 5 is aligned with the sample zone ("B") to be extracted, the contact probe 5 is lowered so that the adhesive/extraction reagent 6 on the tip thereof contacts the sample zone (Fig. 2b). [0033] The adhesive/extraction reagent 6 is selected to readily adhere to the sample zone. Once the adhesive/extraction reagent 6 on the tip of the contact probe 5 contacts the sample zone (Fig. 2b) and the sample zone becomes adhered thereto, the contact probe 5 can be retracted from the contact position (illustrated in Fig. 2b) and moved as shown in Fig. 2c. Since the relative adhesive force of the adhesive/extraction reagent is greater than the adhesive force used to mount the sample on the glass slide, the contact probe 5 pulls the sample zone "B" from the glass slide when withdrawn or retracted. According to one embodiment of the present invention, a glass pipette was used as the

contact probe 5. In this embodiment, the tip of the glass pipette was coated with a solution of piccolyte (568g/l) and xylene (437.5 g/l) by dipping the tip of the glass pipette in the piccolyte/xylene solution.

[0034] In addition to removing the sample zone from the glass slide 2, the contact probe 5 can be used to transfer the extracted sample zone to an analysis container 7 as indicated in Fig. 2c or to any other location, such as a waste container, a culture media, etc. In a preferred embodiment, the contact probe 5 is used to transfer the extracted sample zone to the sample receiving stage of an automated clinical analyzer which is designed to perform a desired analysis of the sample zone. It thus, can be understood that the present invention can provide a fully automated method and system for identifying sample zones on a slide-mounted sample, removing sample zones of interest from the slide-mounted sample, and transporting the extracted sample zones to an automated analyzer which can perform automated analysis of the extracted sample zones.

[0035] In Fig. 2c the extracted sample zone is depicted as being dispensed in a container 7 which, for example, can be a test tube or similar container in which analysis on the extracted sample zone can be initiated or performed. As depicted in Fig. 2c, a reagent solution 8 which removes all or a desired component of the extracted sample zone from the contact probe tip can be placed in the container 7 before the extracted sample zone is deposited therein. For example, in the case of DNA analysis, a solution of Tris (50 mM, pH8.5), EDTA (1mM), Tween 20 (0.5%), and proteinase K (0.2 mg/mL) can be used to remove the extracted sample zone from the tip of the contact probe 5 and dissolve the tissue material for analysis purposes.

[0036] In addition to the contact probe depicted in Figs. 2a-2c, a hollow suction probe could also be used to extract sample zones from the slide-mounted tissue sample 1. Such a suction probe could be provided with sharp annular tip by which sample zones could be punched out and extracted by suction forces.

[0037] Figure 3 is a schematic illustration of an alternative device for extracting sample zones from the slide-mounted tissue sample 1. The extraction device 9 shown in Fig. 3 includes a cutting blade 10 and a grasping arm 11. The grasping arm 11 can be moved in an opposed manner with respect to the cutting blade 10. The grasping arm 11 is shown in its open position in Fig. 3. The grasping arm 11 is movable between the illustrated open position to a closed position in which the tip of the grasping arm 11 contacts the cutting blade 10. The movement of the grasping arm 11 can be controlled by a cable and pulley system in which grasping arm 11 is caused to pivot at its base by applying tension to a cable which passes through a pulley located at the base of the grasping arm 11. The tension on the cable can be applied by actuating a lever or depressing a button 12 on the device which applied tension to the cable in a known manner. Such actuating mechanical structures are known in the art of gripping devices.

[0038] In operating the device of Fig. 3, the cutting blade 10, which is at an obtuse with respect to the central axis of the device can cut out and scoop up a portion of a tissue sample by placing the cutting blade 10 on one edge of a portion of the tissue sample to be extracted and then moving the grasping arm 11 into the closed position. As the grasping arm 11 comes into contact with the tissue sample, it draws the cutting blade 10 into the sample and presses a portion of the sample toward the cutting blade 10 thereby causing a portion of the sample contacted between the cutting blade 10 and the grasping arm 11 to be cut out and scooped up from the sample.

[0039] In a further, Alternative embodiment of the device of Fig. 3, the movement of the grasping arm 11 can be effected by a toothed gear instead of a pulley and a cooperating toothed rod in place of a cable. Such mechanical structures are known in the art of gripping devices.

[0040] Figures 4a and 4b are schematic diagrams of a manual extraction tool manipulator which can be used together with the extraction device of Fig. 3 according to the present invention. In Fig. 4a the extraction tool manipulator is depicted as having a base 13 equipped with a clamping means 14 for removable attaching the device to a brace or support portion of the stage of a microscope (see Fig. 4b). The clamping mechanism includes a clamping plate 15 that is secured to a threaded shaft 16 which passes through a threaded bore 17 in a lower portion of the base 13. A tightening knob 18 is provided on the end of the threaded shaft 16. Turning the tightening knob 18 causes the clamping plate 15 to move with respect to an upper portion 19 of the base 13. Thus, the extraction tool manipulator can be clamped to a portion of the stage of a microscope 20 as depicted in Fig. 4b by positioning a brace or support portion 21 of the stage of the microscope 20 between the clamping plate 15 and the upper portion 19 of the base 13 and turning knob 18 to tighten the clamping plate 15 against the brace or support portion 21 of the stage of the microscope 20.

[0041] The extraction tool manipulator includes a tool holder 22 having a through-bore 23 therein for receiving the shaft of an extraction tool 24. Ideally, the tool holder 22 should allow for damped fore and aft movement of the extraction tool. Therefore, according to a preferred embodiment, the through-bore 23 of the tool holder 22 contains a bushing which can be adjustable tightened against the tool shaft by tool locking screw 24.

[0042] The tool holder 22 is supported by support shaft 25 which is connected at opposite ends by 360° damped swivels 26 and 27 to the tool holder 22 and the base 13. The length of the support shaft 25 between the 360° damped swivels 26 and 27 is adjustable. The adjustment of the independent 360° damped swivels 26 and 27 together with the adjustable length of the support shaft 25 and the position of the tool shaft within through-bore 23, allows a high degree of movement of the extraction tool with respect to a slide-mounted sample positioned on the stage of the microscope. Therefore, an operator can manipulate an extraction tool held by the extraction tool manipulator and remove selected

tissue zones from a slide-mounted tissue sample with a high degree of precision.

[0043] Figure 5 is a functional system diagram which shows how a zone of sample tissue can be directed to an appropriate analysis protocol. As depicted in Fig. 5 a microextraction of a zone of tissue sample can be taken from a slide-mounted tissue sample 1 as discussed above and transferred to a sample preparation stage 28 in which the cells of interest can be extracted and collected for analysis. Excised cells may also be solubilized at this stage. If the cells of interest contain DNA or RNA the extracted sample is subjected to polymerase chain reaction (PCR) amplification and hybridization, strand conformational polymorphism, and southern and northern blotting as desired. If the cells of interest contain proteins, the extracted sample can be subjected to enzyme zymography, an immunoassay, or a biochemical assay.

[0044] Selective extraction or microdissection of frozen tissue sections according to the present invention allows for recovery and analysis of both active enzymes and mRNA. Additionally, the DNA recovered from these sections is in the native condition and can be used for studies such as DNA fingerprinting. Microdissection of paraffin embedded tissues according to the present invention allows for PCR amplification of DNA from pure cell populations representing less than one high powered field, or a single layer of epithelial cells lining cystic spaces.

[0045] For general preparation of samples for frozen section microdissection according to the present invention microdissection slides can be prepared by placing 1% agarose on a standard histology slide and cover slipping. After a short period of time, e.g., 5 minutes the cover slip is removed leaving a thin gel on the slide. A small frozen tissue section, e.g. 25 micron thick, is placed on the agarose gel and briefly stained with eosin. The tissue may also be treated with agents to denature RNase depending on the subsequent extraction method. Under direct microscopic visualization the specific cell population or sub-population of interest is procured from the tissue section utilizing the techniques discussed above.

[0046] For enzyme analysis the procured tissue specimen can be placed in an appropriate buffer depending on the enzyme of interest. The enzyme levels can be measured by several methods including zymography and the use of specific fluorometric substrates. The precise levels of enzyme expression in a specific cell population can be determined.

[0047] For messenger RNA analysis the tissue specimen can be placed on agarose and treated with agents to denature RNase if necessary. The procured tissue specimen is immediately frozen in liquid nitrogen. The tissue can be used immediately or stored at -70°C for several months. The mRNA can be extracted using an oligo dT column (Micro-Fast track mRNA Isolation Kit, Invitrogen Co.). The recovered mRNA of the pure cell populations can be amplified and investigated using PCR technology.

[0048] For DNA analysis the tissue specimen can be placed in a single step extraction buffer solution of 50 mM Tris, pH 8.5; 1mM EDTA, 0.5% Tween 20, and 0.2 mg/ml proteinase K, incubated for four hours at 37°C, followed by ten minutes incubation at 95°C. The recovered DNA can be amplified and analyzed by PCR technology. If native DNA is required for DNA fingerprinting, the proteinase K can be added after DNase.

[0049] For paraffin section microdissection routine formalin fixed, paraffin embedded tissue sections are microdissected after deparafinization and brief staining with eosin. Tissue sections are visualized by direct microscopy and cell populations or subpopulations of interest are procured using a modified glass pipette with the adhesive coated tip discussed above. Tissue specimens as small as one cell can be procured with this method. The specificity of dissection represents a significant improvement over currently known techniques.

[0050] For DNA analysis of paraffin embedded tissue, the glass pipette with the dissected tissue specimen is placed in a single step extraction buffer solution of 50 mM Tris, pH 8.5; 1mM EDTA, 0.5% Tween 20, and 0.2 mg/ml proteinase K which removes the tissue from the pipette tip. Depending on the size of the sample it is incubated from two to twenty-four hours at 37°C, followed by a ten minute incubation at 95°C. The glass pipette tip can then be sterilized and reused.

[0051] Features and characteristics of the present invention will be illustrated by the following examples to which the present invention is not to be considered limited. In the examples and throughout percentages are by weight unless otherwise indicated.

[0052] The following examples were performed in an attempt to establish if the present invention could be used to more specifically study protease distribution during human tumor invasion. Levels of MMP-2 and cathepsin B in fields of invasive breast and colon carcinoma were measured to assess if the enzymes in these regions were quantitatively increased as compared to matched numbers of normal cells from the same patient.

[0053] In the following examples, normal and tumor samples of colon and breast tissue from surgical resections were maintained in a frozen condition (-70°C) until analysis. Tissue section of invasive breast and colon carcinoma were selected based upon histologic evaluation. For the tumor sections histologic fields of tissue which contained invasive tumor and stroma were selected, but not normal epithelium or significant numbers of inflammatory cells. The control sections of normal tissue contained epithelium and a thin section of underlying stroma. The proportion of epithelial and stromal tissue was similar for both normal and tumor sections.

[0054] In the examples microdissection slides were prepared by covering standard histology slides with 200 microliters of warm agarose (1%) and over laying a cover slip. After five minutes the coverslip was removed leaving a thin

bed of agarose on the slide. Twenty micron thick frozen sections were prepared in a cryostat and placed on the agarose gel. The tissue was briefly dipped in eosin. Optimum microdissection was achieved by starting at the edge of each section and systematically dissecting and separating histologic fields of interest with the microdissecting device of Fig. 3. Areas of interest were retained on the slide for subsequent analysis. The DNA content of the specimens was determined by spectrophotometric measurement at 260 nm. The DNA content of each sample was proportional to the number of cells counted in each histologic section.

Example 1

[0055] In this example, samples of normal and tumor tissue matched for cell number were analyzed from each subject. Levels of MMP-2 were determined by zymography and quantified using an Arcus scanner. Results were statistically analyzed using the students t-test. Cathepsin B levels were determined as V_{max} against the substrate Z-Arg-Arg-NHMec.

[0056] The results of this example are set forth in Table 1 below Which lists the cathepsin B activity in matched pairs of invasive colon carcinoma/normal epithelium, and invasive breast carcinoma/normal epithelium. Activity measurement are expressed as V_{max} , nmol/min x mg DNA. Cathepsin B activity was increased an average of 2.3 fold in the colon tumors ($p<0.005$), and 6.9 fold in the breast tumors ($p=0.077$).

TABLE 1

CATHEPSIN B ACTIVITY IN INVASIVE HUMAN COLON CARCINOMA			
SAMPLE	NORMAL	TUMOR	TUMOR/NORMAL
25	1	1.38	4.75
	2	1.89	2.25
	3	1.98	6.32
	4	0.49	1.88
	5	0.44	0.72
	6	1.03	1.92
	7	0.47	1.35
	8	0.19	0.33
	9	1.07	0.90
	10	0.33	0.88
35	Average	0.93	2.13
CATHEPSIN B ACTIVITY IN INVASIVE HUMAN BREAST CARCINOMA			
SAMPLE	NORMAL	TUMOR	TUMOR/NORMAL
40	1	0.63	3.02
	2	0.51	10.08
	3	0.61	4.43
	4	2.21	2.38
	5	2.06	3.72
45	Average	1.20	4.73

[0057] As can be seen from Table 1, all five breast tumors and nine of the ten colon tumors showed increased activity of cathepsin B as compared to matched numbers of normal cells from the same patient (Table 1). Increased activity in the colon tumors ranged from 19% to 283%, with an average increase in tumors of greater than two fold. The increase of cathepsin B activity was more pronounced in breast tumors with an average increase of slightly less than seven fold.

Example 2

[0058] In this example, polymerase chain reaction (PCR) analysis was preformed. On the basis of previously reported cDNA sequences of 72 kDa type IV collagenase, sense and antisense oligonucleotide primers were synthesized for amplification of the enzyme activation site (M. Onisto et al, "Reverse Transcription-Polymerase Chain Reaction Phe-

nototyping of Metalloproteinases and Inhibitors in Tumor Matrix Invasion", *Diagn. Mol. Pathol.*, 2(2):74-80, 1993). The paired oligonucleotide sequences were: 5' - CAA TAC CTG AAC ACC TTC TA, 3' - CTG TAT GTG ATC TGG TTC TTG. Labeled PCR for Single Strand Conformation Polymorphism (SSCP) was obtained by combining the following in a 10 microliter reaction: 1 microliter 10X PCR buffer (100 mM Tris-HCL, pH 8.3; 500 mM KCl; 15 mM MgCl₂; 0.1% w/v gelatin); 1 microliter of DNA extraction buffer; 50 pmol of each primer; 20 nmol each of dCTP, dGTP, dTTP, and dATP; 0.2 microliter [³²P]dCTP (6000 Ci/mmol); and 0.1 unit Taq DNA polymerase. The amplification reaction was carried out for 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

[0059] Figure 6a shows the expression of MMP-2 in ten invasive colon carcinoma cases as compared to normal colonic mucosa from the same patients. The bar graphs show increases of approximately three fold in the 72 kDa pro-form of the enzyme ($p<0.001$) and ten fold in the 62 kDa active form of the enzyme ($p<0.001$).

[0060] Figure 6b shows the expression of MMP-2 in five cases of invasive breast carcinoma. The bar graphs show an appropriate increase of three fold in the 72kDa pro-form of the enzyme ($p<0.05$) and ten fold in the 62 kDa active form of the enzyme ($p<0.05$).

[0061] The 72 kDa pro-type IV collagenase and 62 kDa active form of the enzyme were increased in all ten colon tumors and all five breast tumors as compared to normal tissue from the same patient. The increase was greater in the 62 kDa active form of the enzyme which was elevated an average of ten-fold in both the colon and breast tumors as compared to normal control tissue. The 72 kDa pro-enzyme levels were increased an average of three fold in both tumor types. For both breast and colon tumors the increase in the 62 kDa active enzyme was more variable than that of the pro-enzyme. Elevations in the 62 kDa active enzyme in tumors ranged from 3 to 20 fold while increases in the 72 kDa pro-enzyme were consistently in the 2 to 5 fold range. These results are similar to the recent findings of Davis et al ("Activity of Type IV Collagenases in Benign and Malignant Breast Disease", *Br. J. Cancer*, 67:1126-1131, 1993) in their analysis of human breast tumors. These authors performed zymogram analysis of tissue sections from human breast cancer patients. These analyses demonstrated that the fraction of total MMP-2 present as the 62 kDa activated form was statistically elevated in malignant disease, and a high proportion of this active enzyme species was detected in higher grade tumors. The present invention extends this analysis by comparing and quantitating both 72 kDa and 62 kDa forms of the enzyme in specific regions of invasive tumor and matched normal control epithelium from the same patient.

Example 3

[0062] In this example, strand conformation polymorphism (SSCP) analysis was preformed. Labeled amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide; 20 mM EDTA; 0.05% bromophenol blue, and 0.05% xylene cyanol). The samples were denatured for 5 min at 95°C and loaded onto a gel consisting of 6% acrylamide (49:1 acrylamide:bis), 5% glycerol, and 0.6X TBE. Samples were electrophoresed at 8W at room temperature overnight. Gels were transferred to 3 mm Whatman paper, dried and autoradiography was performed with Kodak X-OMAT film.

[0063] Figure 7 shows SSCP analysis of MMP-2 activation site. The figure shows representative cases of normal colon is mucosa compared to invasive colon carcinoma, and normal breast tissue compared to invasive breast carcinoma. No difference is observed between the normal and tumor specimens. The two band in each lane represent single and double forms of DNA. Similar results were obtained for ten colon carcinomas and four breast carcinomas.

[0064] To assess if increased tumor levels of activated MMP-2 are due to a mutation in the enzyme, PCR was used to amplify DNA sequence coding for the activation site of gelatinase A from the colon and breast tumors. The activation site is located 10 kDa from the N-terminus of the enzyme and contains the site of cleavage which converts the 72 kDa pro-enzyme into the 62 kDa active species. Amplification and analysis of this region by PCR and SSCP showed no detectable mutations in any of the ten colon tumors or four breast tumors studied. These results suggest that increased levels of active enzyme in invasive tumors is most likely due to a tumor associated activating species. The sensitivity of PCR amplification of DNA from microdissected frozen tissue sections was determined to be less than one high power field. Similar to the amplification of DNA, amplification of mRNA from small cell populations was preformed according to the present invention using reverse PCR.

[0065] A previous study indicated that MMP-2 is up-regulated in human colon carcinoma. However, recently several studies using *in situ* hybridization analysis report that the mRNA level of MMP-2 in human colon carcinoma is increased in the stromal cells as opposed to the tumor cells. In order to address this possibility frozen tissue sections were microdissected to measure enzyme levels of MMP-2 in separate tumor and stromal cell populations. From a single high power field sufficient tissue was recovered to quantitate enzyme levels by zymography. Studies of invasive tumor cells and adjacent stroma from three cases indicate that 72 kDa pro-MMP-2 and active 62 kDa form are associated with both tumor cell and stromal cell populations. Preliminary data suggest that the highest enzyme levels are at the tumor-stromal interface.

[0066] The above results indicate that microdissection of frozen tissue sections allows for more specific analysis of

cell populations within human tumors than by conventional techniques. Microdissection may be used in combination with a number of different technologies that allow for analysis of enzymes, mRNA and DNA from pure populations or subpopulations of particular cell types. This simple technique may have utility in characterizing protease distribution during human tumor invasion, precisely determining protease expression in tumor and/or stromal cell populations as

5 an indicator of tumor aggressiveness, and monitoring the effectiveness of anti-protease therapeutic agents in inhibiting protease activity at the tumor-stromal interface. In addition, combination of this microdissection technique with PCR, RT PCR, differential display and SSCP may identify genetic alterations in specific subpopulations of tumor or stromal cell that would not be evident in heterogeneous human tumor samples.

[0067] Although the present invention has been described with reference to particular means, materials and embodiments, from the foregoing description, one skilled in the art can easily ascertain the essential characteristics of the present invention and various changes and modifications may be made to adapt the various uses and characteristics without departing from the scope of the present invention as described by the claims which follow.

15 **Claims**

1. A method of direct extraction of cellular material from a tissue sample comprising :

- 20 a) providing a slide-mounted tissue sample having a plurality of zones of cells;
- b) forming an image field of cells of said tissue sample utilizing a microscope;
- c) identifying at least one zone of cells of interest in said image field of cells, the at least one zone of cells of interest having a different type of cell than an adjacent zone of cells;
- d) extracting by microdissection said at least one zone of cells of interest from said tissue sample by:

- 25 d1) i) contacting the at least one zone of cells of interest with an adhesive-tipped probe which has a contact surface area which is equal to or no greater than a surface area of the at least one zone of cells of interest, wherein the relative adhesive force of the adhesive tipping said probe is greater than the adhesive force of the one zone of cells of interest on said slide; and then
- d1) ii) retracting the adhesive-tipped probe from the position of contact with the tissue sample, thereby causing said at least one zone of cells of interest in the image field of cells attached to the contact surface area of the adhesive-tipped probe to be pulled away by microdissection;

before finally :

- e) analyzing said extracted at least one zone of cells of interest.

35 2. The method of claim 1, wherein said adhesive is an adhesive/extraction reagent comprising a solution of piccolyte in xylene.

40 3. The method of claim 1 or 2, wherein said at least one zone of cells of interest comprises a plurality of the same type of cell.

45 4. The method of any of the preceding claims, wherein said at least one zone of cells of interest comprises a single cell.

50 5. The method of any one of the preceding claims, wherein said extracting said at least one zone of cells of interest from said tissue sample is automated.

6. The method of any one of the preceding claims, wherein said analyzing is performed by an automated analyzer.

7. The method of claim 5, wherein said extracted zone of cells of interest is automatically transferred to said automated analyzer.

8. The method of any one of the preceding claims, wherein said extracted zone of cells is analyzed for enzymes, antigens, DNA or RNA.

55 9. The method of any one of the preceding claims, wherein said image is displayed on a display monitor.

10. The method of any one of the preceding claims, wherein said tissue sample is stained prior to forming the image field and said staining is used to identify said at least one zone of cells of interest from said image field of cells.

11. The method of claim 10, wherein said at least one zone of cells of interest is identified from said image field of cells by an automated contrast identification method.

5 12. A method of direct extraction of cellular material from a tissue sample according to claim 1, wherein said at least one zone of cells of interest consists of a member selected from the group consisting of RNA, DNA and proteins.

Patentansprüche

10 1. Verfahren zur direkten Extraktion von Zellmaterial aus einer Gewebeprobe, umfassend:

- a) das Vorsehen einer auf einem Objekträger angebrachten Gewebeprobe mit einer Vielzahl von Zonen von Zellen;
- b) die Bildung eines Bildfeldes von Zellen dieser Gewebeprobe unter Verwendung eines Mikroskops;
- 15 c) das Identifizieren mindestens einer Zone von Zellen, an welchen ein Interesse besteht, in diesem Bildfeld der Zellen, wobei die mindestens eine Zone der Zellen, an welchen ein Interesse besteht, eine andere Zellart aufweist als eine benachbarte Zone von Zellen;
- d) das Extrahieren dieser mindestens einen Zone von Zellen, an welchen ein Interesse besteht, aus dieser Gewebeprobe durch Mikrodissektion durch:

20 d*i*) Kontaktieren der mindestens einen Zone von Zellen, an welchen ein Interesse besteht, mit einer an der Spitze mit Klebstoff versehenen Sonde, die einen Kontaktflächenbereich hat, der gleich oder nicht größer als ein Flächenbereich der mindestens einen Zone von Zellen ist, an welchen ein Interesse besteht, wobei die relative Klebestärke des Klebstoffs an der Spitze dieser Sonde größer ist als die Klebestärke der einen Zone von Zellen, an welchen ein Interesse besteht, auf dem Objekträger; und nachfolgendes

25 d*ii*) Zurückziehen der an der Spitze mit Klebstoff versehenen Sonde aus der Position des Kontakts mit der Gewebeprobe, wodurch bewirkt wird, daß die mindestens eine Zone von Zellen, an welchen ein Interesse besteht, im Bildfeld der Zellen, die am Kontaktflächenbereich der an der Spitze mit Klebstoff versehenen Sonde haften, durch Mikrodissektion weggezogen werden; bevor schließlich

30 e) die extrahierte mindestens eine Zone von Zellen, an welchen ein Interesse besteht, analysiert wird.

2. Verfahren nach Anspruch 1, wobei der Klebstoff ein Klebstoff/Extraktionsreagens ist, das eine Lösung von Piccolyt in Xylol umfaßt.

35 3. Verfahren nach Anspruch 1 oder 2, wobei die mindestens eine Zone von Zellen, an welchen ein Interesse besteht, eine Mehrzahl von Zellen derselben Art enthält.

4. Verfahren nach einem der vorhergehenden Ansprüche, wobei die mindestens eine Zone von Zellen, an welchen ein Interesse besteht, eine einzige Zelle enthält.

40 5. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Extrahieren der mindestens einen Zone von Zellen, an welchen ein Interesse besteht, aus der Gewebeprobe automatisiert ist.

45 6. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Analysieren auf einem automatisierten Analy-
sator durchgeführt wird.

7. Verfahren nach Anspruch 5, wobei die extrahierte Zone von Zellen, an welchen ein Interesse besteht, auf den automatischen Analysator transferiert wird.

50 8. Verfahren nach einem der vorhergehenden Ansprüche, wobei die extrahierte Zone von Zellen im Hinblick auf Enzyme, Antigene, DNA oder RNA analysiert wird.

9. Verfahren nach einem der vorhergehenden Ansprüche, wobei das Bild auf einem Display-Monitor angezeigt wird.

55 10. Verfahren nach einem der vorhergehenden Ansprüche, wobei die Gewebeprobe vor der Bildung des Bildfeldes gefärbt wird und die Färbung zur Identifizierung der mindestens einen Zone von Zellen, an welchen ein Interesse besteht, im Bildfeld der Zellen verwendet wird.

11. Verfahren nach Anspruch 10, wobei die mindestens eine Zone von Zellen, an welchen ein Interesse besteht, im Bildfeld der Zellen durch ein automatisiertes Kontrast-Identifikationsverfahren identifiziert wird.

5 12. Verfahren zur direkten Extraktion von Zellmaterial aus einer Gewebeprobe nach Anspruch 1, wobei die mindestens eine Zone von Zellen, an welchen ein Interesse besteht, aus einem Mitglied ausgewählt aus der Gruppe bestehend aus RNA, DNA und Proteinen besteht.

Revendications

10

1. Méthode d'extraction directe de matériel cellulaire d'un échantillon de tissu comprenant :

a) la fourniture d'un échantillon de tissu, monté sur lame, ayant plusieurs zones de cellules ;

b) la formation d'un champ d'image de cellules dudit échantillon de tissu en utilisant un microscope ;

15

c) l'identification d'au moins une zone de cellules intéressante dans ledit champ d'image de cellules, la au moins une zone de cellules intéressante ayant des cellules d'un type différent de celui de la zone de cellules adjacente ;

d) l'extraction par microdissection de ladite au moins une zone de cellules intéressante de l'échantillon de tissu par :

20

dl) i) la mise en contact de la au moins une zone de cellules intéressante avec une sonde à embout adhésif ayant une surface de contact égale ou non supérieure à la surface de la au moins une zone de cellules intéressante, la force d'adhérence relative de l'adhésif constituant l'embout de ladite sonde étant supérieure à la force d'adhérence de la zone de cellules intéressante sur ladite lame et ensuite

25

dl) ii) le retrait de la sonde à embout adhésif de la position de contact avec l'échantillon de tissu, ce qui entraîne l'arrachage par microdissection de ladite au moins une zone de cellules intéressante dans le champ d'image de cellules collée à la surface de contact de la sonde à embout adhésif;

avant enfin :

30

e) l'analyse de ladite au moins une zone de cellules intéressante extraite.

2. Méthode selon la revendication 1, dans laquelle ledit adhésif est un réactif adhésif/d'extraction comprenant une solution de piccolyte dans du xylène.

35

3. Méthode selon la revendication 1 ou 2, dans laquelle ladite au moins une zone de cellules intéressante comprend plusieurs cellules du même type.

40

4. Méthode selon une quelconque des revendications précédentes, dans laquelle ladite au moins une zone de cellules intéressante comprend une cellule unique.

5. Méthode selon une quelconque des revendications précédentes, dans laquelle ladite extraction de ladite au moins une zone de cellules intéressante dudit échantillon de tissu est automatisée.

45

6. Méthode selon une quelconque des revendications précédentes, dans laquelle ladite analyse est réalisée par un analyseur automatique.

50

7. Méthode selon la revendication 5, dans laquelle ladite zone de cellules intéressante extraite est transférée automatiquement dans ledit analyseur automatique.

55

8. Méthode selon une quelconque des revendications précédentes, dans laquelle des enzymes, des antigènes, des ADN ou des ARN sont analysés dans ladite zone de cellules intéressante extraite.

9. Méthode selon une quelconque des revendications précédentes, dans laquelle ladite image s'affiche sur un moniteur.

10. Méthode selon une quelconque des revendications précédentes, dans laquelle ledit échantillon de tissu est coloré avant formation du champ d'image et ladite coloration est utilisée pour identifier ladite au moins une zone de cellules intéressante dans ledit champ d'image de cellules.

EP 0 748 439 B1

11. Méthode selon la revendication 10, dans laquelle ladite au moins une zone de cellules intéressante est identifiée dans ledit champ d'image de cellules par une méthode d'identification par contraste automatisée.

5 12. Méthode d'extraction directe de matériel cellulaire d'un échantillon de tissu selon la revendication 1, dans laquelle ladite au moins une zone de cellules intéressante comprend un élément choisi dans le groupe constitué par les ARN, les ADN et les protéines.

10

15

20

25

30

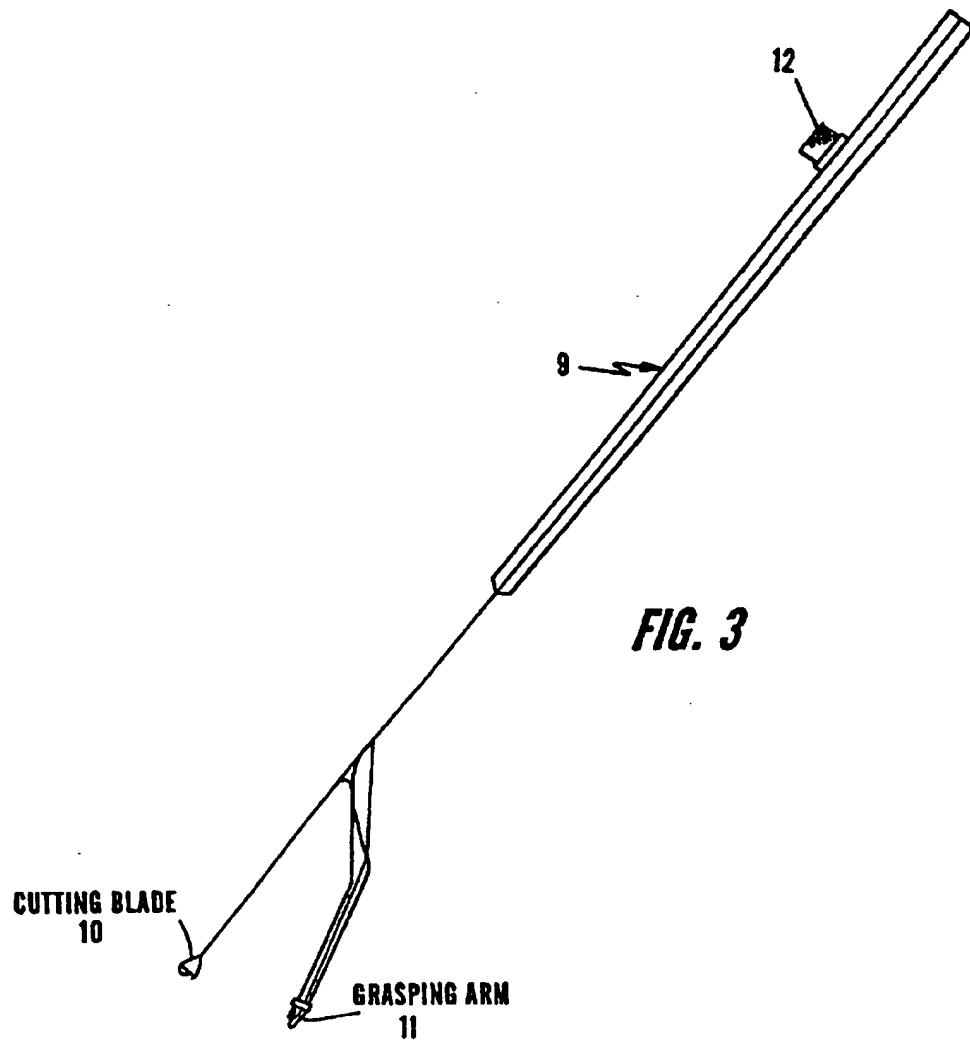
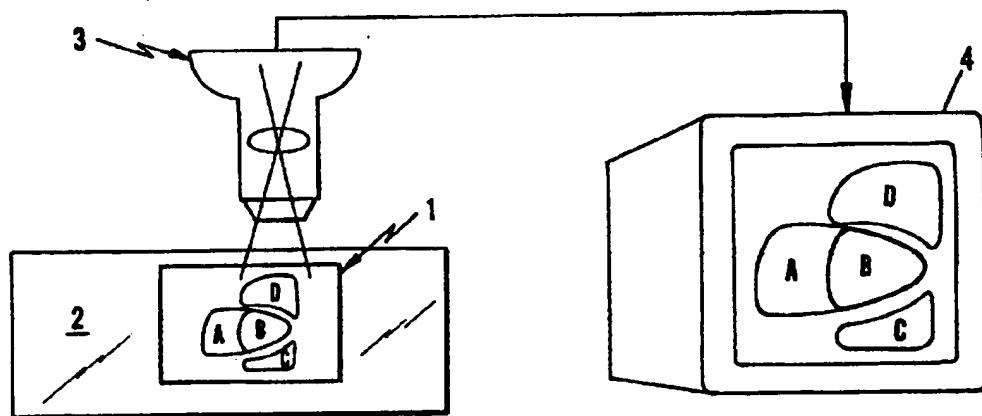
35

40

45

50

55



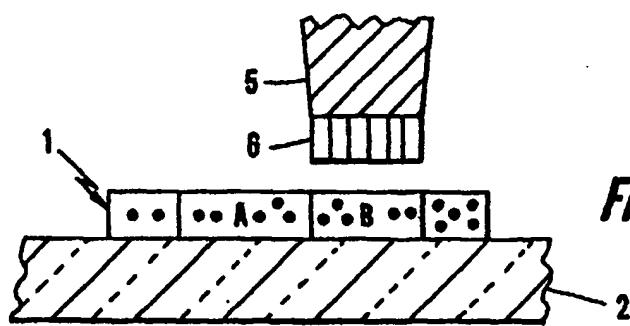


FIG. 2a

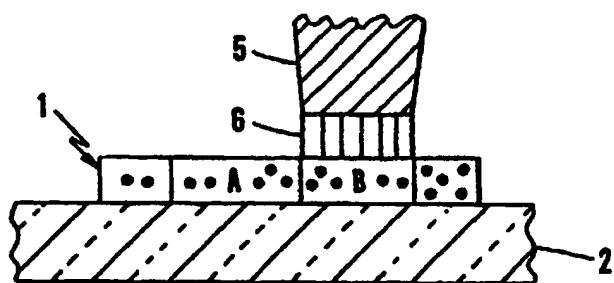


FIG. 2b

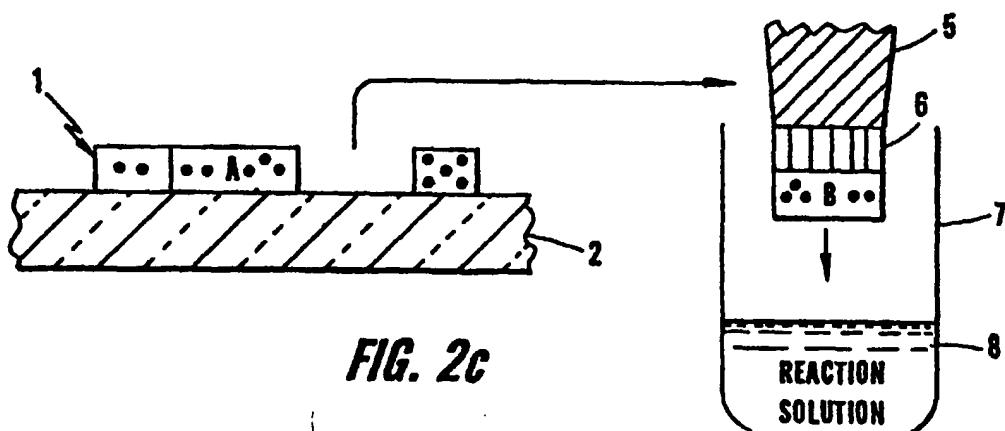
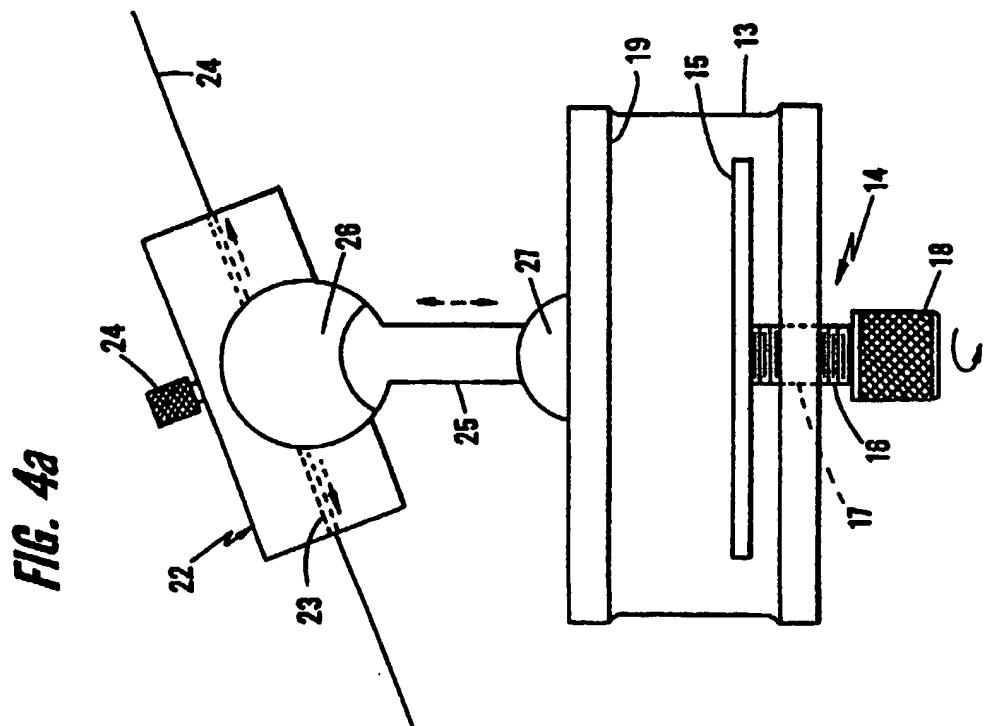
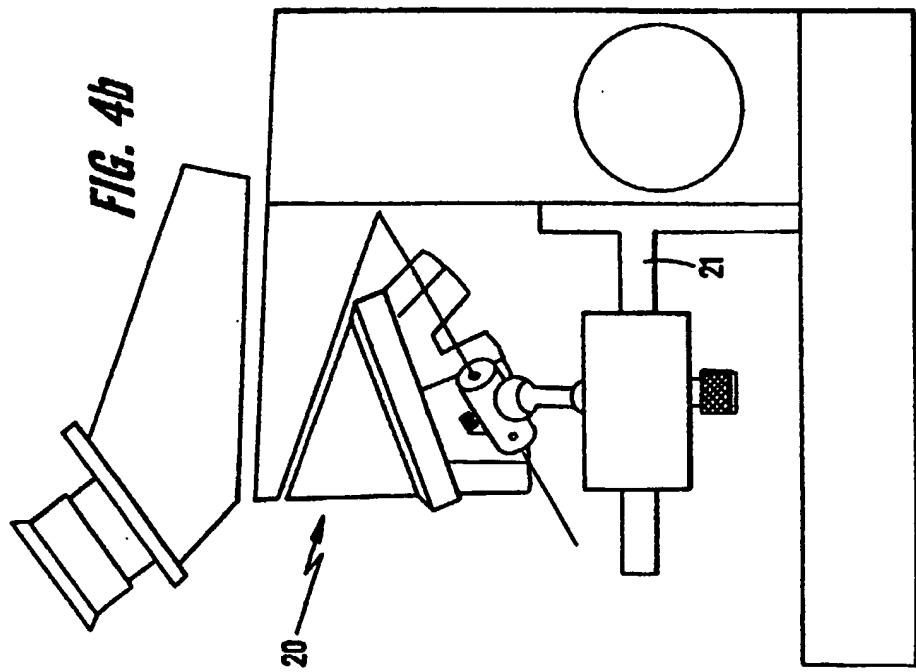


FIG. 2c



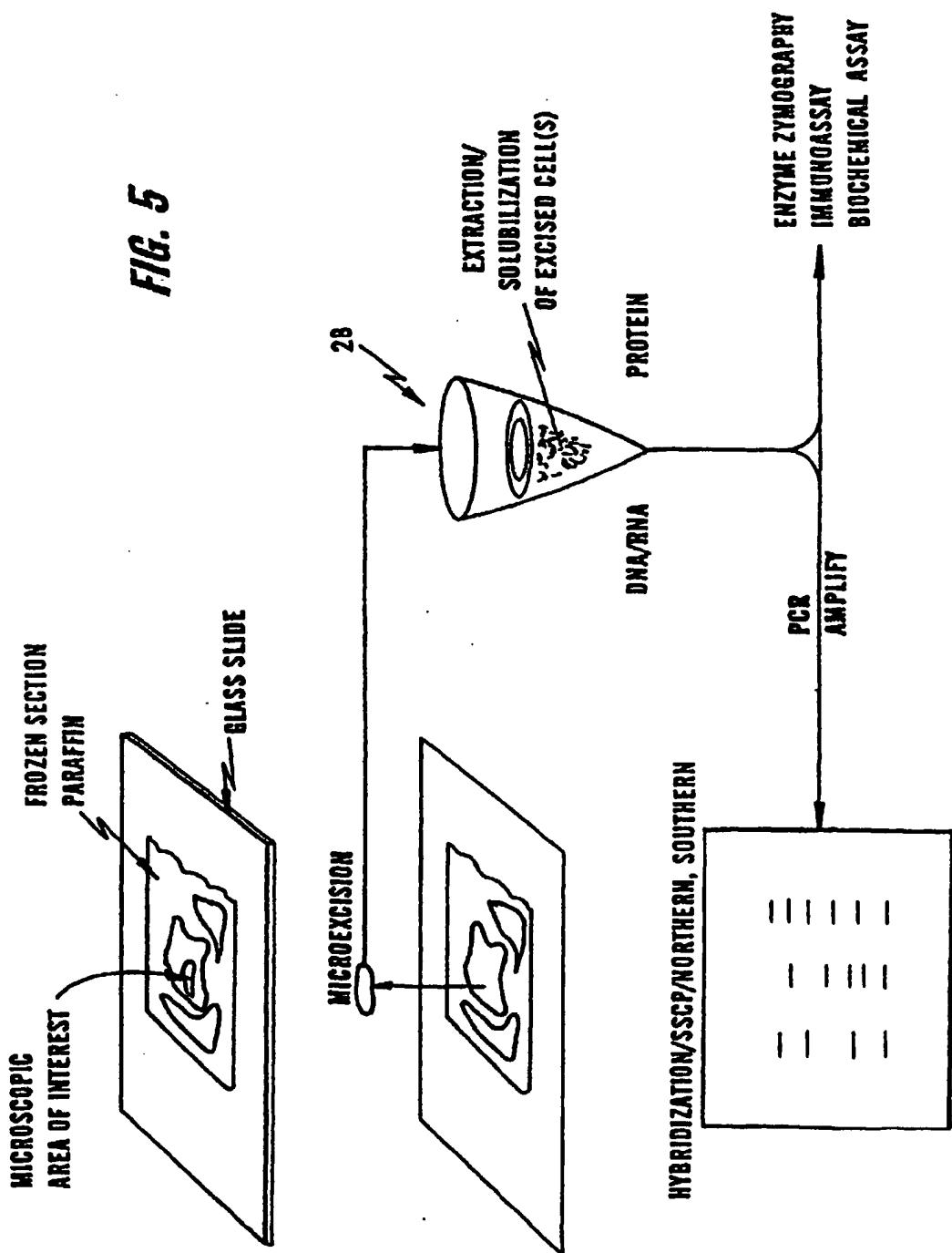


FIG. 6a

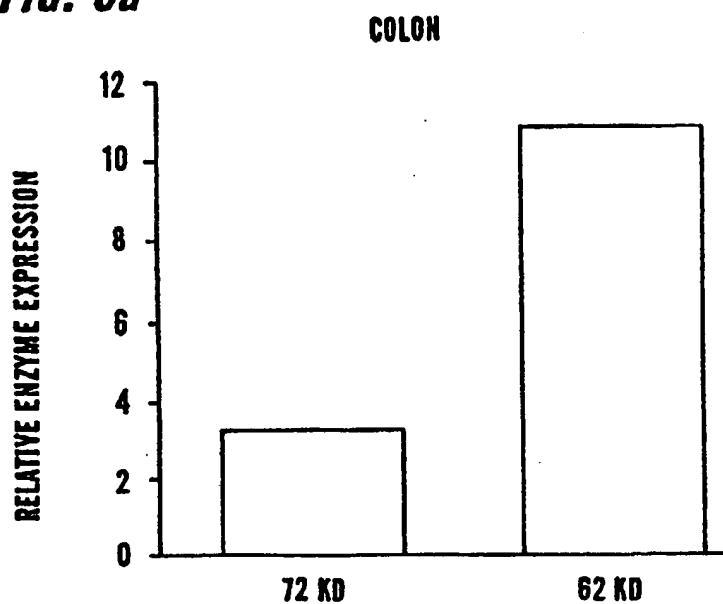
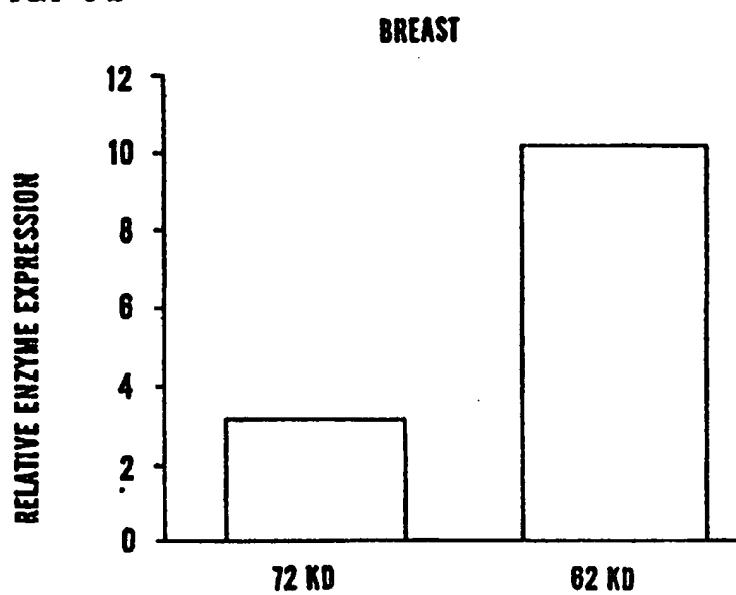


FIG. 6b



Colon Breast

N T N T

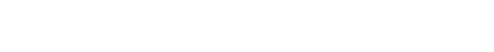


FIG.7